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Transgenic Tobacco Plants Expressing Pea Chloroplast *Nmdh* cDNA in Sense and Antisense Orientation[1]

Effects on NADP-Malate Dehydrogenase Level, Stability of Transformants, and Plant Growth

Maria Faske, Jan E. Backhausen, Martina Sendker, Marielle Singer-Bayrle, Renate Scheibe, and Antje von Schaewen*

Pflanzenphysiologie, FB 5, Biologie/Chemie, Universität Osnabrück, D-49069 Osnabrück, Germany

A full-length cDNA encoding light-activated chloroplast NADP-malate dehydrogenase (NADP-MDH) (EC 1.1.1.82) from pea (*Pisum sativum* L.) was introduced in the sense and antisense orientation into tobacco (*Nicotiana tabacum* L.). Transgenic plants with decreased or increased expression levels were obtained. Because of substantial age-dependent differences in individual leaves of a single plant, standardization of NADP-MDH levels was required first. Then, extent and stability of over- or under-expression of *Nmdh*, the gene encoding NADP-MDH, was characterized in the various transformants. Frequently, cosuppression effects were observed, indicating sufficient homology between the endogenous tobacco and the heterologous pea gene. Analysis of the T_1 and T_2 progeny of a series of independent transgenic lines revealed that NADP-MDH capacity ranged between 10% and ≥10-fold compared with the wild type. Under ambient conditions whole-plant development, growth period, and fertility were unaffected by NADP-MDH reduction to 20% of the wild-type level; below this threshold plant growth was retarded. A positive growth effect was registered in young plants with stably enhanced NADP-MDH levels within a defined developmental window.

In plants MDHs catalyze the reversible pyridine-dinucleotide-dependent interconversion between OAA and malate in various cellular compartments. NAD-dependent isoforms are present in mitochondria, microbodies, and the cytosol (Gietl, 1992), whereas chloroplasts possess an NADP-dependent enzyme (NADP-MDH, EC 1.1.1.82) that is subject to posttranslational light/dark modulation mediated by the Fd/thioredoxin system (Scheibe, 1987).

In contrast to C_4 photosynthesis, in which NADP-MDH plays a well-known role in CO_2 prefixation (Edwards et al., 1985), the function of the enzyme in C_3 plants is less clear, since no example of a similar role in carbon assimilation is known. Here NADP-MDH probably fulfills other tasks. There is evidence that the enzyme is involved in fine-tuning of the stromal redox state (Scheibe, 1987; Backhausen et al., 1994) via the so-called "malate valve": the controlled reduction of OAA is able to poise the ATP/NADPH ratio inside the chloroplast when alterations in electron use or ATP levels occur, which are caused by changes in light intensity or by stomatal opening. A large part of the malate formed in the light is due to NADP-MDH activity. Mediated by the malate-OAA shuttle, malate, and concomitantly reducing equivalents, can be transferred from the chloroplasts into the cytosol (Heber, 1974; Heineke et al., 1991).

In plant-cell metabolism malate claims a central role for various reactions (Lance and Rustin, 1984). The export of reducing power into the cytosol is assumed to be involved in nitrate assimilation (House and Anderson, 1980; Champigny, 1995), to drive mitochondrial respiration (Raghavendra et al., 1994), and to stabilize the cytosolic pH (Davies, 1986). Furthermore, malate can be stored in the vacuole (Winter et al., 1994). Together with the anaplerotic CO_2-fixation reaction catalyzed by PEP carboxylase (Melzer and O'Leary, 1987; Lepiniec et al., 1994), malate can account for up to 15% of the ^{14}C-labeled metabolites in spinach leaves at the end of the light period (Gerhardt and Heldt, 1984). Malate is also transported from the leaf into the root system, where it can provide electrons and serve as the carbon skeleton for N assimilation (Lee, 1980), or be involved in the uptake of nutrient salts (Smirnof and Stewart, 1985).

To gain insight into the role of light-activated NADP-MDH in C_3 plants, we obtained transgenic tobacco (*Nicotiana tabacum* L.) plants with either increased or decreased levels of this chloroplast enzyme. A cDNA encoding NADP-MDH from pea (*Pisum sativum* L.), driven by the constitutive cauliflower mosaic virus 35S promoter, was introduced into tobacco in sense or antisense orientation. Transformants with reduced or increased NADP-MDH levels were identified and expected to provide a useful tool with which to study the effects on regulatory processes and plant-growth behavior.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tobacco (*Nicotiana tabacum* L. var Xanthi) seeds were a kind gift from the laboratory of M.J. Chrispeels (Depart-

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* Corresponding author; e-mail schaewen@sfbbio1.biologie.uni-osnabrueck.de; fax 49-541-969-2870.

Abbreviations: NADP-MDH, NADP-dependent malate dehydrogenase; OAA, oxaloacetate.

ment of Biology, University of California, San Diego). After tissue culture, wild-type plants and transformants (T_0) were grown in commercial soil mixture (10% sand, 10% pumice, 10% loam, 35% compost, and 35% peat) in growth chambers. Up to d 30 after germination the pot size was 5 cm in diameter (70 mL), and from then on it was 14 cm in diameter (1.3 L). After potting the plants were transferred to their final growth regimes. In the growth chamber light intensity was 250 to 350 μmol quanta m^{-2} s^{-1} at plant height for a daily period of 16 h of light (22°C), 8 h of darkness (18°C), and 75% RH. Greenhouse facilities were used during the summer months (June through September of 1995). The average daily temperature was 20.4°C and the average daily sunshine period was 9.6 h. Peak light intensities at plant height ranged between 200 and 1400 μmol quanta m^{-2} s^{-1}.

Seeds of the transgenic progeny and control plants were surface sterilized using a method communicated by A. Rodrigues-Franco (Facultad de Ciencias, Universidad de Cordoba, Spain). Up to 100 seeds were spread evenly inside a closed, sterile, 1.5-mL microfuge tube and placed inside of a microwave oven along with a 1-L Erlenmeyer flask filled with 800 mL of cold water, and set twice for 7 min at 650 to 700 W (in between, seeds were allowed to cool for 2 min and the water was replaced). Sterilized seeds were spread on solidified (0.8% agar) Murashige and Skoog medium (Sigma) containing 2% Suc and 250 mg L^{-1} Claforan (cefotaxime sodium, Duchefa, Haarlem, The Netherlands) supplemented with 100 mg L^{-1} kanamycin (medium A) or without kanamycin (medium B). After 3 d in the dark at 4°C for synchronizing germination, further cultivation was performed in growth chambers for about 7 d at 70 μmol quanta m^{-2} s^{-1} for a daily period of 16 h of light (24°C) and 8 h of darkness (20°C). Seedlings were then transferred to soil and grown as described above.

For assessing whole-plant growth, surface-sterilized seeds of selected transgenic lines (T_1 and T_2 progeny) were germinated on sterilized sand that was moistened with water. After 10 d seedlings were planted in commercial soil mixture and grown as described above. For interpolation of biomass development, the scored data were fitted by a fifth-order polynomial regression using the software package GRAFIT version 3.0 (Erithacus Software Ltd., Staines, UK).

Cloning Procedures

Plasmids containing the complete cDNA sequence coding for NADP-MDH from pea (*Pisum sativum* L.) were constructed as shown in Figure 1, starting from two cDNA clones termed *r-mdh* and *t-mdh* (W. Reng, Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, Germany). The insert of clone *r-mdh* encodes the mature part of the enzyme and clone *t-mdh* encodes the missing 5′ end, comprising the plastidic transit peptide plus part of the mature N terminus (Reng et al., 1993). The complete cDNA sequence was assembled in vector pASK40 (Skerra, 1989) and termed *g-mdh*. From this construct, the 1,356-bp *Eco*RI fragment was purified, blunt-ended, and inserted into the *Sma*I site in plant-expression vector pA35S (Höfte et al., 1991), flanked by the cauliflower mosaic virus 35S promoter and octopine synthase termination sequences. Sense or antisense orientation of the cDNA fragments was verified by *Hin*dIII restriction digests (Fig. 1). Both plant-expression cassettes containing the complete *Nmdh* cDNA in either sense or antisense orientation were introduced as 2,161-bp *Eco*RI/*Hin*dIII fragments into the T-DNA region of binary vector pDE1001 (Denecke et al., 1990). Two different *Agrobacterium tumefaciens* C58C1 strains, LBA4404 and GV2260 (Deblaere et al., 1985), were transformed with the sense- and antisense-pDE1001 con-

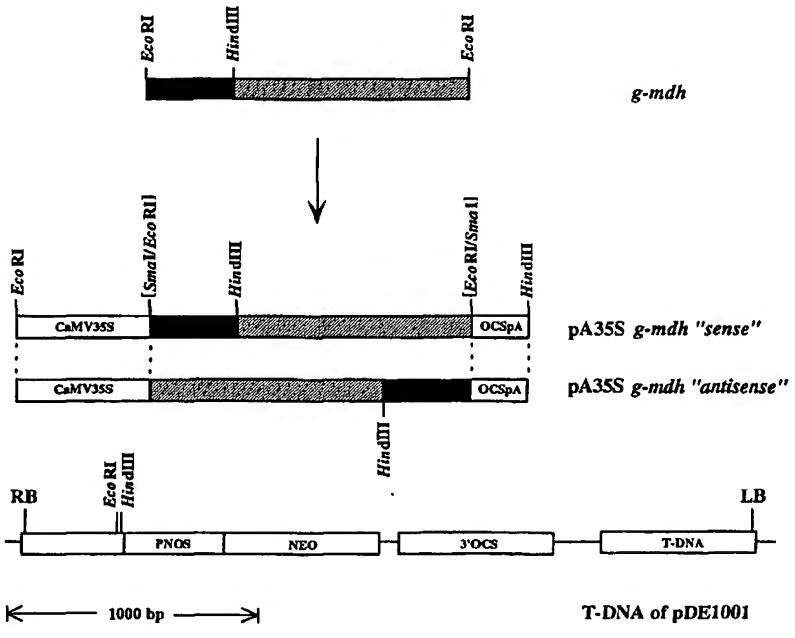

Figure 1. Construction scheme of plasmids with *Nmdh* in sense and antisense orientation. The complete cDNA fragment (1356 bp, coding for pea NADP-MDH and its transit peptide) was inserted between the cauliflower mosaic virus (CaMV35S) promoter and the octopine synthase (OCS) termination sequences of plant expression cassette pA35S (Höfte et al., 1991). The 2161-bp *Eco*RI/*Hin*dIII fragments of constructs pA35S *g-mdh* sense and pA35S *g-mdh* antisense were inserted into the respective *Eco*RI/*Hin*dIII sites of binary vector pDE1001 (Denecke et al., 1990). RB/LB, Right and left T-DNA borders, respectively; PNOS, nopaline synthase promoter driving the neomycin phosphotransferase gene (NEO); and hatched box, cDNA fragment used as a probe for hybridization experiments.

structs using the freeze-thaw method described by Höfgen and Willmitzer (1988) and used for cocultivation of tobacco leaf discs.

Leaf-Disc Transformation and Regeneration of Transgenic Plants

A. tumefaciens-mediated transformation of tobacco leaf discs followed the procedure described by Voelker et al. (1987). Combined callus and shoot induction was on solidified (0.8% agar) Murashige and Skoog medium containing 2% Suc, supplemented with 0.1 mg L^{-1} naphthalene acetic acid and 1 mg L^{-1} benzylaminopurine. For selection of transformed shoots Claforan (500 mg L^{-1}) and kanamycin (100 mg L^{-1}) were included. After 6 to 8 weeks regenerated shoots were transferred to kanamycin-supplemented agar (medium A). Shoot cuttings that formed roots were planted in soil and grown for at least 3 weeks before the determination of NADP-MDH capacity. As controls, kanamycin-sensitive (i.e. pseudo-wild-type) and wild-type tobacco plants were regenerated in parallel, the latter by omitting kanamycin selection (medium B).

Preparation of Leaf Extracts

To reduce stress effects resulting from tissue culture, plants were grown for at least 3 weeks in soil before experiments were started. The youngest leaf measuring about 1.5 cm in length was defined as the first leaf of an individual plant. The third leaf was about 30% of the size of a fully expanded source leaf. The complete third leaf of each plant was ground in liquid nitrogen and the frozen powder was subsequently homogenized in 500 μL of degassed extraction medium: 50 mM Tris-HCl, 1 mM EDTA, 14 mM 2-mercaptoethanol, 0.01% (w/v) BSA, 0.5% (v/v) Triton X-100, and 1% (w/v) polyvinylpolypyrrolidone, pH 8.0. Under these conditions degradation and/or inactivation of NADP-MDH was undetectable. Aliquots for measuring chlorophyll content were withdrawn, and the remaining samples were stored in liquid nitrogen for determination of NADP-MDH activity and protein concentration.

Determination of NADP-MDH Activities

For routine measurements of NADP-MDH activity in the third leaf (counted from the top) of individual plants, preparation of leaf extracts was simplified as follows: two leaf discs of 1 cm in diameter were cut with a cork borer from both sides of the midrib (central area) and ground under liquid nitrogen in a 1.5-mL microfuge tube. The frozen powder was suspended in 200 μL of extraction medium as described above, but without polyvinylpolypyrrolidone. To determine maximal NADP-MDH activity (i.e. NADP-MDH capacity), the enzyme was fully activated by incubation for 20 min in the presence of 100 mM DTT (reduced form, dissolved in 100 mM Tris-HCl, pH 8.0) and 0.1% (w/v) BSA. NADP-MDH activity was measured at 340 nm in a standard assay mixture, as described by Scheibe et al. (1986). Plants that were used for growth analyses were preselected according to samples taken 30 d after germination. Final categorization was based on measurements at the time of harvest.

For the estimation of in vivo NADP-MDH activities, samples were taken using the freeze-clamp method and extracted according to Scheibe and Stitt (1988). For freeze-clamping, an LCA4 gas-exchange system (ADC, Hoddesdon, UK) with a specially modified PLC2 leaf chamber was used (fine-mechanic shop of the Universität Osnabrück, Germany).

Chlorophyll concentration was determined according to Arnon (1949) before centrifugation of the samples. Total soluble protein was measured in cleared extracts using the method of Bradford (1976) with BSA as a reference protein.

Estimation of Molecular Masses and NADP-MDH Protein Contents

Discontinuous SDS-PAGE of protein extracts (Laemmli, 1970) was conducted in a vertical minigel system (Mini-Protean II, Bio-Rad), including a molecular weight standard mixture (Dalton Mark VII-L, Sigma).

Preparation of protein blots with subsequent immunodetection was essentially as described by Graeve et al. (1994). Extraction of total protein was in 50 mM Hepes-NaOH, pH 7.0, 0.1% (w/v) SDS, 2 mM sodium bisulfite, and 0.01% (w/v) BSA. A polyclonal rabbit antiserum, raised against purified pea NADP-MDH, was used for the detection of NADP-MDH from pea and tobacco. Relative amounts were estimated from a dilution series.

Nucleic Acid Analyses

Genomic DNA was prepared from frozen leaf tissue as described by Dellaporta et al. (1983). Total RNA was isolated from the fourth leaf (counted from the top) of individual plants according to the method of Logemann et al. (1987). Nucleic acids were blotted from agarose gels onto Nytran membranes (Schleicher & Schuell). DNA- and RNA-blot analyses were conducted using standard techniques (Sambrook et al., 1989). For an estimation of signal strength, dot-blot filters with serial dilutions of pea cDNA clone *r-mdh* were prepared essentially as described by von Schaewen et al. (1995). The blots were probed with radioactively labeled cDNA fragments (970 bp) of plasmid *r-mdh*, purified from agarose gels using the Geneclean-II kit (BIO 101, San Diego, CA). Labeling of DNA fragments with [α-^{32}P]dCTP and removal of nonincorporated nucleotides was as described previously (von Schaewen et al., 1995).

RESULTS

Assessment of NADP-MDH Capacity in Wild-Type and Pseudo-Wild-Type Tobacco Plants

To analyze the distribution of NADP-MDH in tobacco wild-type plants, maximal enzyme activity (i.e. capacity) was monitored in discs cut from every leaf of individual plants during a period of 3 to 9 weeks after germination. The results shown in Figure 2 demonstrate that in tobacco leaves NADP-MDH capacity depends strictly on plant age

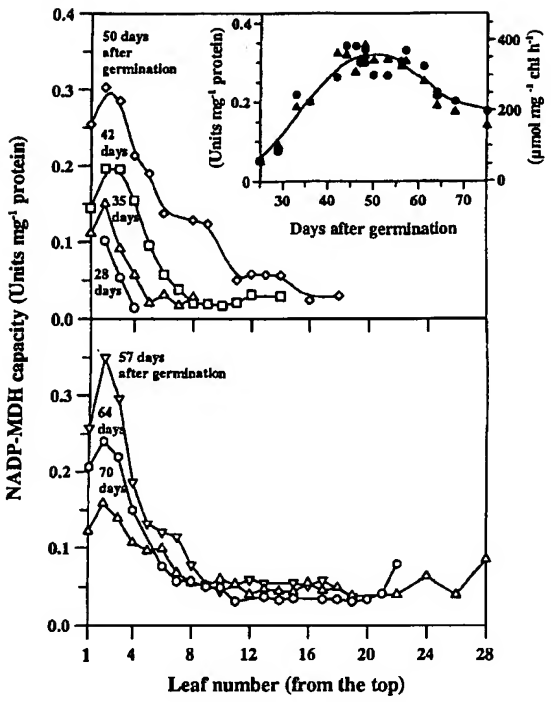

Figure 2. NADP-MDH activities in individual leaves of tobacco wild-type plants. Graphs are based on the mean values obtained from two plants for each time point. The inset in the top panel shows the calibration curve of NADP-MDH capacities in third-leaf samples. Tobacco wild-type and pseudo-wild-type plants (after regeneration from leaf discs in tissue culture) were assayed over the indicated time period. The values are either based on protein (left scale, ●) or chlorophyll (right scale, ▲) contents. chl, Chlorophyll.

and on the developmental state of the tissue. Enzyme activity in young, developing leaves was about 5- to 10-fold higher than in older, fully expanded leaves. This held true for a correlation based on protein content (Fig. 2), and also for a correlation of NADP-MDH capacity with leaf area or chlorophyll content (data not shown).

Especially in young leaves of wild-type tobacco plants NADP-MDH capacity varied substantially over the entire growth period (Fig. 2). To correlate the enzyme activities in third-leaf samples of the transformants with those of wild-type plants grown in parallel, NADP-MDH capacities were determined at various times after transfer to soil. The time-dependent differences of endogenous NADP-MDH capacities in third-leaf samples of pseudo-wild-type regenerants paralleled the result found for wild-type plants, regardless of whether the data were related to protein or to chlorophyll contents (calibration curve; Fig. 2, inset). Therefore, in all further experiments untransformed cv Xanthi wild-type plants were grown in parallel and used as controls.

Analysis of Primary Transformants (T_0 Plants)

From 33 independent transformants expressing pea *Nmdh* in sense orientation, obtained after infection with two different *A. tumefaciens* strains (L preceding the number of the transformant refers to LBA4404, $n = 11$; P refers to GV2260, $n = 22$), only four showed significant overexpression of *Nmdh* (the data for two of them are shown in Figure 3A). It was confirmed that the more than 2-fold NADP-MDH capacity in third-leaf samples of these transformants (e.g. L12) correlated with an equally enhanced signal on RNA blots (qualitative estimation, data not shown). Frequently, cosuppression of endogenous tobacco *Nmdh* was observed (Jørgensen, 1991), which indicates sufficient homology between the endogenous tobacco and the

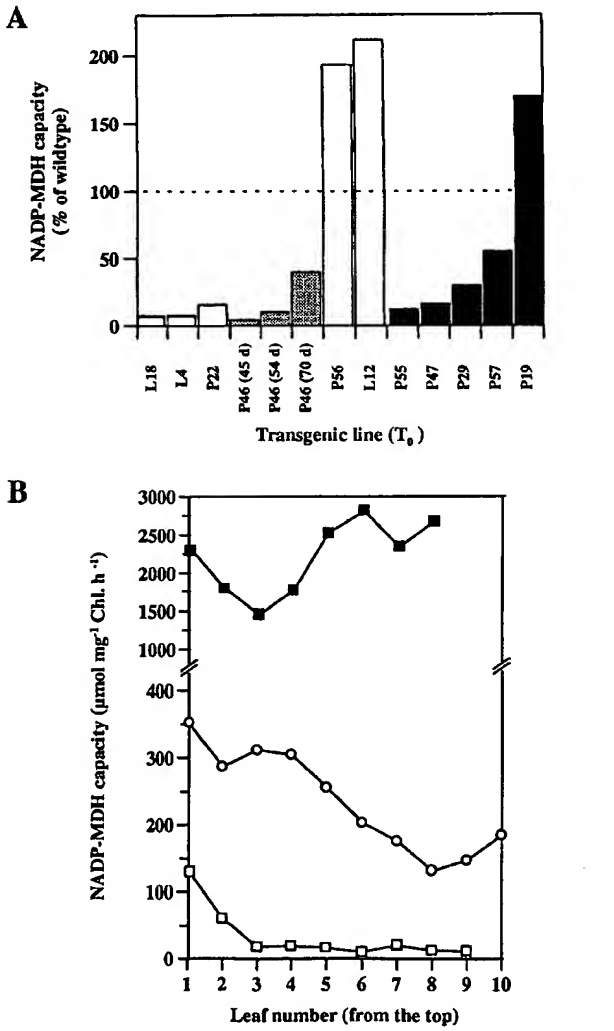

Figure 3. NADP-MDH capacities in the third leaf of selected transformants. A, Activities of the primary transformants (T_0) are shown as a percentage of those determined in third-leaf samples of tobacco wild-type plants. The calibration curve shown in Figure 2 was used as a reference (100%). A total of 36 antisense and 50 sense transformants was analyzed. Open and shaded bars, Sense plants; black bars, antisense plants. Multiple measurements were done between d 30 and 70 after transfer of plants to soil. B, NADP-MDH capacity in individual leaves of two stable L18-T_1 plants at d 43 postgermination, showing either strong overexpression (L18-T_1-B6/OE, ■) or strong cosuppression (L18-T_1-B5/UE, □) of NADP-MDH. O, Untransformed control plant (wild type). Chl, Chlorophyll.

heterologous pea gene. A recent report shows that sequences with only 71% homology can give successful cosuppression (Trevanion et al., 1997). Some of the sense transformants showed a decrease of NADP-MDH capacity to less than 20% of the wild-type level in third-leaf samples (Fig. 3A). For transformants L18, L4, and P22 this effect was constant over the entire growth period. Similarly low NADP-MDH levels were not stable in all cosuppressed transgenic sense lines. Transformant P46, for example, showed the strongest initial reduction in NADP-MDH capacity (about 5% of the wild type). However, when assaying the third leaf at d 54 and 70 after germination, levels had increased to 40% of NADP-MDH wild-type activities (Fig. 3A, gray bars).

Expression of the heterologous pea *Nmdh* cDNA in antisense orientation resulted in stable reduction of NADP-MDH capacity in transgenic tobacco plants, but antisense transformants with NADP-MDH levels that were reduced to less than 13% of the wild-type level were not obtained (Fig. 3A; Table I). After selfing most of the T_1-antisense plants showed NADP-MDH activities ranging between 50 and 100% of the wild-type level in the third leaf. The reason that one of the antisense transformants (P19) showed elevated NADP-MDH activity, which was also transmitted to the T_1 progeny (Table I), is obscure and was not analyzed further.

Quantitation of the T-DNA Integrations

Southern-blot analysis of transgenes alone is not sufficient to reveal the exact copy number of T-DNA integrations (Masle et al., 1993). To determine the number of independent T-DNA insertions in the dihaploid tobacco genome, seeds of the selfed transformants (T_1 progeny) were germinated on agar containing kanamycin. The percentage of germinating seeds was always equal to the proportion of germinated seeds without kanamycin selection. The deduced T-DNA copy numbers for selected transgenic lines are shown in Table I. For some of the transformants an estimation of one to two T-DNA integrations per genome is based on 10 to 15% kanamycin sensitivity of the T_1 progeny. Deviations from the theoretically expected value were already found in a similar study (Kilby et al., 1992) and had been ascribed to a non-Mendelian behavior of the kanamycin-resistance gene.

Characterization of T_1 Plants

For studies concerning whole-plant growth under various conditions, a high number of near-isogenic plants with stably suppressed or increased NADP-MDH levels was required. For a better comparison, T_1 individuals from different transgenic lines were categorized according to their NADP-MDH capacities into "overexpressors" (>200%), wild-type-like, and "underexpressors" (<40%). Among the selfed progeny of some transgenic sense lines (e.g. transformant L18), most T_1 individuals revealed either low (<20%) or high (>2-fold) NADP-MDH levels (Table I), which were stable in all leaves during the entire growth period (data not shown). Individuals were defined as stable when the NADP-MDH content in all available leaves was either at least 3-fold higher or less than 20% of the wild type (grown in parallel) during the entire growth period (Fig. 3B).

NADP-MDH activities in third-leaf samples of strong overexpressors were 4- to 5-fold higher compared with wild-type plants, and immunologically cross-reacting material on protein blots was increased even more (up to

Table I. *Characteristics of the various transgenic lines*

Inferred T-DNA copy numbers and NADP-MDH capacities in the third leaf of selected transformants (T_0) are compared with the distribution of NADP-MDH capacities in individual T_1 plants. WT, Tobacco wild type. T-DNA copy numbers were estimated from the percentage of kanamycin-resistant seedlings (kan^R) in the T_1 progeny of the selfed transformants (n = 50).

Line	T-DNA Copies	NADP-MDH Level			
		Relative MDH level in T_0 plants	Range of MDH levels in kan^R T_1 progeny (no. of plants)		
			<40	±100	>200
			% of WT		
Sense					
L18	2	7.5	10	8	32
L4	1	8	0	44	6
P22	1–2	16	42	3	5
P46	(1)	5, 10, 40[a]	28	18	4
L12	1–2	210	0	0	50
Antisense					
P55	2	13	0	50	0
P47	2	17	3	47	0
P29	1	30	0	50	0
P57	2	55	0	50	0
P41	1	70	0	50	0
P19	1	170	0	25	25

[a] NADP-MDH capacity determined at d 45, 54, and 70 after germination.

20-fold). This estimation includes a 45-kD form in addition to the 43-kD unit length NADP-MDH band, which is also specifically recognized by the pea antiserum in overexpressors (Fig. 4C, lane OE). Subsequent experiments with isolated chloroplasts from wild-type and L18-B6-overexpressor plants revealed that at comparable levels of intactness (76 and 75%, respectively), NADP-MDH capacity and protein content in the overexpressor are elevated 5-fold compared with the wild type (data not shown). Therefore, we speculate that the 45-kD cross-reacting polypeptide most likely represents unprocessed NADP-MDH precursors in the cytosol, which are recognized by the antiserum but do not contribute to measurable activity.

Southern-blot analysis of *Hin*dIII-digested genomic DNA of individual L18-T_1 plants suggests that cosuppression effects ensue from higher T-DNA copy numbers when compared with the situation in overexpressors. Different

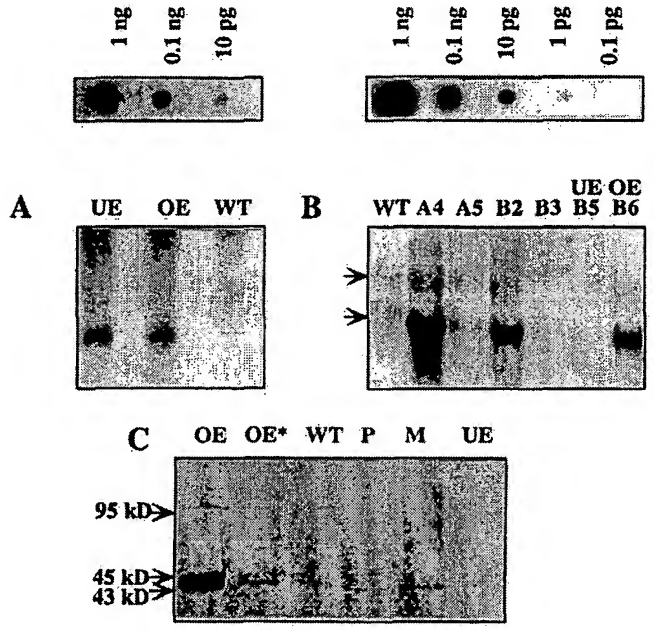

Figure 4. Analysis of *Nmdh* expression in leaf samples of L18-T_1 plants. A, For Southern blots, genomic DNA from wild type (WT), selected underexpressors (UE), or overexpressors (OE) was prepared. Ten-microgram aliquots were digested with *Hin*dIII (compare Fig. 1), separated in 0.8% agarose gels, and transferred to nylon membranes. B, RNA blots were run with 20 μg of total RNA under denaturing conditions. Southern and northern blots were hybridized with ^{32}P-labeled 970-bp *Hin*dIII fragments of pea cDNA clone *r-mdh* (Reng et al., 1993; compare Fig. 1). Signal strengths were estimated from dot-blot standard filters developed in parallel. The two arrows mark the positions of weak signals recognized by the heterologous pea probe in wild-type tobacco plants. C, Immunodetection of NADP-MDH on protein blots. Third-leaf samples were separated in a 15% SDS-polyacrylamide gel and transferred to nitrocellulose. A polyclonal rabbit antiserum raised against the purified pea enzyme was used to detect tobacco and pea NADP-MDH polypeptides. Lane M, Molecular mass standards plus 1 μg of recombinant pea NADP-MDH purified from *Escherichia coli*, essentially as described by Reng et al. (1993). Leaf extracts of pea (P), L18-T_1-B6 (OE), L18-T_1-B5 (UE), and a tobacco wild-type plant (WT) were analyzed, each equivalent to 200 μg of soluble protein. OE*, Dilution of L18-T_1-B6 extract equivalent to 20 μg of protein.

signal intensities were observed by hybridization of th 1,125-bp internal *Hin*dIII-fragment (Fig. 4A, compare lane UE and OE) with the radiolabeled 970-bp probe (Fig. 1 Fragments larger than 5000 bp were detected whe genomic DNA was digested with *Eco*RI (one restriction sit within the introduced T-DNA construct; data not shown This indicated that multiple T-DNA integration in direc tandem repeat at one locus had not occurred.

Using RNA-blot analysis of total RNA, endogenous an heterologous *Nmdh* mRNA could not be distinguished (Fi 4B). In wild-type tobacco samples two mRNA species hy bridized weakly with the heterologous cDNA probe fro pea, whereas strong labeling of a single band was observe with RNA samples of individual overexpressors. Hig mRNA levels always correlated qualitatively with hig NADP-MDH capacities (Fig. 3B) and protein contents (Fi 4C) in individual overexpressing L18-T_1 plants, where *Nmdh* signals of underexpressing L18-T_1 plants on RN and protein blots were below the detection limit whe compared with wild-type controls (Fig. 4, B and C).

In heterotrophic plastids NADP-MDH activity is unde tectable. Because of constitutive expression of the tran gene, root extracts of L18-T_1 overexpressors showed rela tively high NADP-MDH capacities (0.8–2.7 units mg^{-} protein). In root extracts of L18-T_1 underexpressors, how ever, NADP-MDH activity was, as in wild-type plants, a the detection limit (0.017 $\pm$ 0.005 unit mg^{-1} protein), whic indicates that in transgenic line L18 cosuppression operate at the DNA level. In contrast, relatively high NADP-MDH capacity (i.e. DTT-dependent, about 0.15 unit mg^{-1} pro tein) was detected in root extracts of underexpressing T plants of line P46. In this case, the cosuppression effe observed in leaves seems to depend on the tissue-specifi expression of the endogenous tobacco *Nmdh* gene.

Growth Analysis of T_1 and T_2 Individuals in the Greenhouse

Possible correlations of plant-growth parameters wit altered NADP-MDH capacities in transgenic plants wer analyzed in comparison with the wild type. Because i several lines multiple insertion events probably occurre (Table I), T_1 and T_2 individuals from eight different tran genic sense lines were used for the first set of experiment For growth-rate analyses, plants were preselected accord ing to their NADP-MDH capacities at d 30 before they wer transferred to the greenhouse. A total of 360 individual (including one-third of the untransformed wild-type plant and one unstable line) was analyzed in three sets. Ran domly selected plants were harvested in 10- to 14-d inte vals for determination of fresh and dry weights, leaf are specific leaf weight, and NADP-MDH activity. For a bette comparison, transgenic plants were classified according t their NADP-MDH capacities into overexpressors (>300% wild type), underexpressors (<20% wild type), or wild type-like plants.

For untransformed wild-type control plants, the typica developmental change of the NADP-MDH activities, a described above (compare Fig. 2), was observed. Highes NADP-MDH capacities were scored during the period c

Table II. *Stability of NADP-MDH capacity in transgenic tobacco sense lines used for growth analysis*

NADP-MDH activities were first determined after 35 d in the greenhouse and again at harvest. The categorization is based on NADP-MDH capacities of untransformed control plants grown in parallel (100%): underexpressors (UE, <50%), overexpressors (OE, >200%), and wild-type plants (50–200%). A subset of stable transformants was used for scoring the data compiled in Figure 5.

Transgenic Line	No. of Individuals				
	Total no.	<50%	50–200%	>200%	Unstable
P8-T_1	13	0	13	0	0
P14-T_1	14	0	12	0	2
L-6-T_1	14	0	5	9	0
L12-T_1	39	0	5	30	4
L18-T_1	42	6	4	19	13
L18-T_2 (B5/UE)	49	16	0	0	33
L18-T_2 (B6/OE)	36	0	0	35	1

maximal growth (d 40–60). Analysis of the various transgenic lines that were used in these experiments revealed significant differences, not only in their NADP-MDH expression levels, but also in their stability of NADP-MDH expression. For classification, the NADP-MDH capacity of the wild-type plants at harvest was taken as 100%. Individuals in which NADP-MDH activity deviated from the initial score at d 30 were denoted as unstable and were not included in the following growth analyses (Table II).

Stable transgenic plants that were examined during the phase of maximal growth (around d 50 after germination) showed a significant correlation of their fresh and dry weights with their respective NADP-MDH capacities (Table III). Extreme underexpressors exhibited reduction in leaf area and shoot fresh or dry weights, whereas for overexpressors these parameters increased. The observation that specific fresh and dry weights (i.e. weight per leaf area) remained unaltered indicated that no change in leaf morphology had occurred. The dry weight data were used to interpolate a continuous time course of biomass accumulation (Fig. 5). The curve fit indicates that plants with a high NADP-MDH capacity developed faster and reached their final weight earlier than the wild type, whereas plants with a reduced enzyme capacity lagged behind. The differences were visible only during maximal plant growth and disappeared later in development.

Because the effect on plant growth was found for three independent transgenic lines, we consider it unlikely that the described growth phenotype is merely the result of T-DNA insertion (or insertions) at a certain locus in the tobacco genome. In short-term experiments, steady-state photosynthetic parameters, levels of other pace-making Calvin-cycle enzymes, and metabolite contents of the leaves were not significantly altered (data not shown). Apart from the differences in growth, few phenotypic deviations occurred. Some of the transformants had narrower leaves, but when assaying the T_1 progeny this did not strictly correlate with the NADP-MDH contents and can probably be attributed to tissue-culture aftereffects (Masle et al., 1993).

Growth Analysis under Climate-Controlled Conditions

To minimize effects possibly caused by the noncontrolled environmental conditions during growth in the greenhouse, T_2 plants from the most stable overexpressing (L18-T_1-B6) and underexpressing lines (L18-T_1-B5) were chosen for further growth analyses under climate-controlled conditions. After 30 d a clear correlation between NADP-MDH content and growth rate was detected. Transgenic plants with less than 20% of NADP-MDH capacity were restricted in growth, whereas overexpressors showed a faster accumulation of biomass than the corresponding wild-type plants. The effects are obvious from data scored with shoot tissue (leaves plus stem) and are

Table III. *Comparison of growth parameters*

Growth parameters were determined for wild-type tobacco and stable transgenic sense plants as given in Table II. The transgenic plants showed either extreme over- or underexpression of NADP-MDH. The data were scored during the period of maximal plant growth in the greenhouse using total overground tissue (leaves plus stem) and are the means ± SD. The average values obtained for untransformed plants (wild type) represent 100% (n = 6).

Growth Parameter	Underexpressors	Wild Type	Overexpressors
NADP-MDH capacity (μmol mg^{-1} chlorophyll h^{-1})	19 ± 10	100 ± 17	507 ± 100
Leaf area (m^2)	73 ± 7	100 ± 8	130 ± 6
Shoot fresh weight (g)	70 ± 8	100 ± 12	125 ± 16
Shoot dry weight (mg)	54 ± 7	100 ± 8	118 ± 9
Specific leaf weight (fresh) (g m^{-2})	106 ± 9	100 ± 7	95 ± 6
Specific leaf weight (dry) (mg m^{-2})	105 ± 5	100 ± 10	107 ± 10

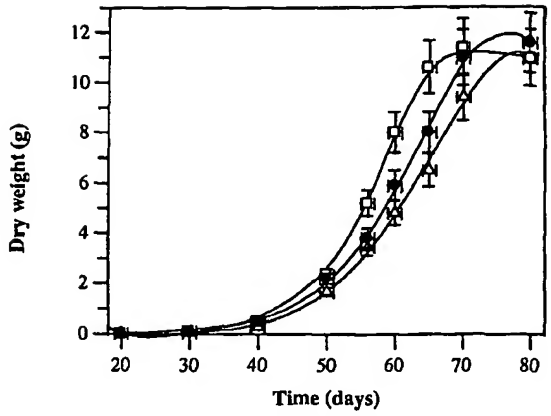

Figure 5. Graphic representation of dry weight accumulation over time. Plants were grown in the greenhouse and harvested at the indicated times. A subset (≤20% and ≥300%) of the transgenic plants used for growth analyses (Tables II and III) was compiled. The horizontal error bars indicate that the harvesting procedure took up to 3 d. The scored data were fitted by a fifth-order polynomial regression, using the software package GRAFIT version 3.0. □, Overexpressors; ●, untransformed wild-type plants; and △, underexpressors.

less clear with root tissue (Fig. 6), which is difficult to sample from soil-grown plants.

As already observed in the greenhouse for *Nmdh*-overexpressing plants, at the end of a prolonged growth period the enhancement in biomass production was no longer apparent. After 70 d in soil no significant differences could be detected for *Nmdh* overexpressors on a whole-plant basis compared with the wild type (Fig. 6). This was also found for selected individuals of other lines (L12 and P46). Between over- and underexpressing individuals, other striking differences became evident from the activation states of NADP-MDH (Table IV). In wild-type plants about 35% of the available enzyme is activated in vivo under the chosen growth conditions. Overexpressors reached slightly lower activation states (expressed as percentage), which means that their actual NADP-MDH activity was three times higher compared with the wild type. In contrast, underexpressors activated a large part (70–80%) of their available NADP-MDH, which, however, corresponds to only about 20% of the actual wild-type activity (Table IV). Similar observations were made when *Sorghum bicolor* NADP-MDH was overexpressed in the C_4 plant *Flaveria bidentis* (Trevanion et al., 1997).

DISCUSSION

Several recently conducted physiological studies using transgenic plants, for example, with reduced levels of regulatory Calvin-cycle enzymes (Paul et al., 1995; Price et al., 1995) or of the plastidic phosphate translocator (Riesmeier et al., 1993; Heineke et al., 1994), are based on the assumption that mRNA or protein levels, once defined for a distinct sample, remain constant in all leaves during the entire growth period. This seems to ignore the possibility of tissue-specific, age-dependent, or stress-induced endogenous control mechanisms, which could influence both the enzyme levels in wild-type plants and transgene expression in the transformants.

For the transgenic approach described here, the aim was to generate significant differences in NADP-MDH activity that remain stable during the entire life cycle of the plant. NADP-MDH activities were monitored in leaf samples of wild-type tobacco plants during the growth period. This revealed that between different leaves of individual plants, the maximal activities (i.e. NADP-MDH capacities) varied by a factor of about 10 (Fig. 2), without regard to whether the data were related to chlorophyll or protein content or to leaf area. Further variations were registered over time (see inset in Fig. 2). During maximal growth of the wild-type plants, NADP-MDH levels were about six times higher compared with the beginning or the end of the life cycle. Thus, in experiments that addressed the importance of an enzyme with respect to plant growth, the physiological and

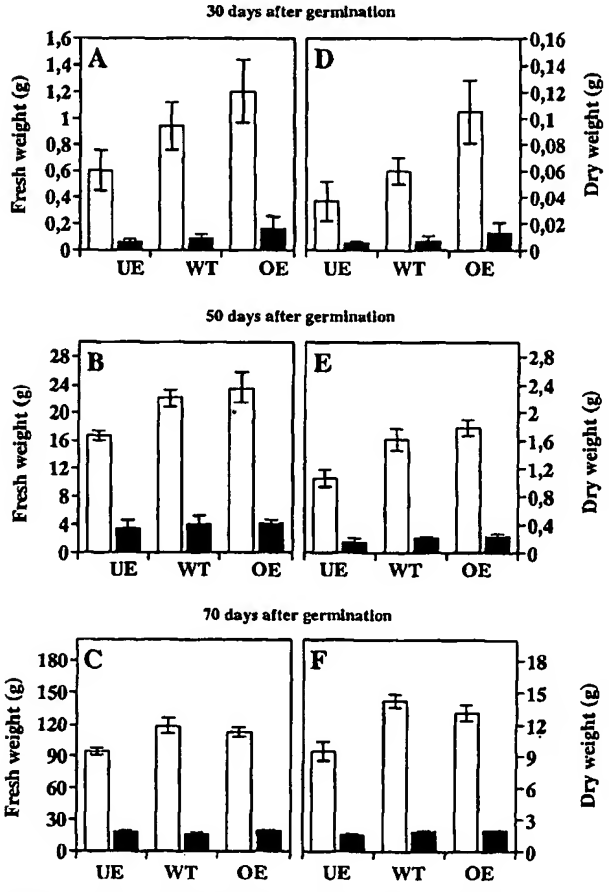

Figure 6. Growth analysis of L18-T_2 plants. Data from the T_2 progeny of selfed L18-T_1-B6 overexpressor and L18-T_1-B5 underexpressor plants are compared with those of untransformed tobacco wild type. Plants were grown at a light intensity of 250 to 350 μmol quanta m^{-2} s^{-1} (at plant height) in a growth chamber as described in "Materials and Methods." Fresh (A–C) and dry weights (D–F) of total shoot (open bars) and root tissue (filled bars) were determined at d 30 (A and D), d 50 (B and E), and d 70 (C and F) after germination. Each bar represents the mean from four individual plants; the range of deviation is indicated by a vertical line.

Table IV. *Comparison of NADP-MDH capacities and the respective activation states in leaf extracts*

Wild-type and individual stable T_2 plants of selected transgenic lines were grown in a climate chamber at 350 μmol quanta m^{-2} s^{-1}. After 55 d samples were taken 2 h after illumination using the freeze-clamp method (see "Materials and Methods"). The number of plants analyzed in the different categories varied: wild type, $n = 9$; underexpressors (<20% of wild type), $n = 4$; overexpressors (>300% of wild type), $n = 7$.

NADP-MDH	Underexpressors (L18-T_2-B5)	Wild Type	Overexpressors (L18-T_2-B6)
Capacity (μmol mg chlorophyll^{-1} h^{-1})	12 ± 5	130 ± 16	507 ± 100
Activation state (% of capacity)	72 ± 12	34 ± 8	26 ± 9
Actual activity (μmol mg chlorophyll^{-1} h^{-1})	8.7	44.0	131.0

developmental variations during the plant's life span, which for NADP-MDH occur in a range (6- to 10-fold change) that is often described as the result of successful plant transformation, must be taken into account. For the evaluation of altered enzyme levels in the different transformants, the corresponding data obtained with wild-type plants, therefore, served as a reference.

The *Nmdh* cDNA from pea was chosen for two reasons. First, overexpression is more effective with heterologous transgenes, and cosuppression effects are less frequently observed (Jørgensen, 1990). Second, it has been shown previously that silencing of endogenous genes can be similarly achieved by transformation with closely related but nonidentical sequences (e.g. Phe ammonia lyase from bean in transgenic tobacco plants [Elkind et al., 1990]; and, recently, NADP-MDH from *S. bicolor* in transgenic tobacco plants [Trevanion et a., 1997]). A number of sense transformants with strongly cosuppressed NADP-MDH activity were found, indicating sufficient homology between the endogenous and heterologous sequences. However, no good antisense effect was observed (Table I), a fact that could be explained by a weaker homology in the 5′ region that comprises the chloroplast signal sequences, since the complete pea cDNA was used for both sense and antisense constructs (Fig. 1). In most but not all cases described, extensive pairing of sense and antisense transcripts is required to efficiently block expression of the endogenous gene by triggering degradation of the double-stranded RNA molecules (Nellen and Lichtenstein, 1993).

T_1 individuals showing either extreme overexpression or extreme cosuppression effects were found among the selfed progeny of three independent transgenic lines (P22, P46, and L18). The mechanisms responsible for the high variation in *Nmdh* expression levels seem to differ between the transgenic lines (Table I), which can be explained by: (a) integration of two T-DNA copies at different (L18) or linked loci (L12); (b) copy number and/or tissue-specific effects on the introduced transgene, operating at different levels (DNA or mRNA, as indicated by different *Nmdh* expression in roots of L18 versus P46 individuals); and (c) transgene inactivation by methylation (Meyer, 1995; Matzke et al., 1996), which could account for the observation of kanamycin-resistant plants with near-wild-type levels (Table I) or developmentally unstable individuals (Table II) (Hobbs et al., 1990).

Despite the exact nature of the effects, categorization of selected individuals into groups with either stably enhanced (overexpressor, >2-fold) or reduced (underexpressor, <40%) NADP-MDH capacities, compared with the wild type (Table I), allowed for simplification of the following experiments. The possibility that only tissue-culture effects influenced *Nmdh* expression can be ruled out, because transformants with near-wild-type activities (i.e. pseudo-wild-type regenerants) showed the same leaf- and age-dependent pattern of NADP-MDH capacities as the wild-type plants. For individual transformants that showed either of the two extremes of stable *Nmdh* expression (Fig. 3B), correlation with the respective mRNA and protein contents was additionally confirmed by gel-blot analyses (Fig. 4). In strong NADP-MDH overexpressors (≥5-fold of the wild-type) a 45-kD band was reproducibly immunodetected on protein blots that could reflect precursor-polypeptide accumulation in the cytosol. In a different study efficient uptake and processing of NADP-MDH from a C_4 plant (*S. bicolor*) was observed in transgenic tobacco plants with up to 2-fold-enhanced enzyme levels (Gallardo et al., 1995).

The main interest of this work, in addition to the characterization of some genetic aspects of the transformants and of the effects on *Nmdh* expression levels, was to analyze the physiological consequences caused by altered NADP-MDH capacities in the mutant plants. The requirement for a minimal amount of this enzyme was already evident from the initial transformant screen, since a reduction to less than 5 to 7% of the wild type was not observed (Table I). Furthermore, in the selfed progeny of line L18 activities beyond this level were never observed in strongly cosuppressed individuals (Table IV). Possibly, this level defines a threshold of NADP-MDH activity needed for plant viability under the given growth conditions. Similar results were obtained upon expression of a homologous *Nmdh* antisense construct in potato. Transformants with NADP-MDH activity of less than 10% of the wild-type levels were not found (B. Müller-Röber, personal communication).

As a first approach toward a physiological analysis, the effect of altered *Nmdh* expression on plant growth was studied. The analysis focused on selected T_1 and T_2 lines (namely L6, L12, L18, P8, P14, and P46), which showed the highest numbers of stable over- or underexpressing individuals. The NADP-MDH levels in these lines ranged between 10% and 6-fold of the wild-type level. In all lines analyzed a correlation between plant growth and NADP-MDH levels was observed under both near-natural (greenhouse; Fig. 5) and climate-controlled (growth chamber; Fig.

6) conditions. The curve fit of the dry weight data shown in Figure 5 shows that the overexpressors developed (i.e. grew and aged) faster, whereas the growth of the underexpressors was retarded.

To this end, the exact nature of the interactions between NADP-MDH and plant growth remains unclear. For example, higher stromal NADP-MDH contents can lead to elevated export rates of reducing equivalents into the cytosol (Krömer, 1995). Malate can either be used for cytosolic NADH production, as required for nitrate reduction, or for mitochondrial ATP production to drive cytosolic Suc synthesis. The altered enzyme-activation states (Table IV indicate that the NADPH/NADP ratio must be higher in the chloroplast stroma of the underexpressors and considerably lower in the overexpressing plants. This is probably caused by altered rates of malate formation and export. An optimized ATP/NADPH ratio in the chloroplast stroma is one possibility to explain the positive effect of increased NADP-MDH activity, whereas a limitation of malate formation in underexpressors seems to retard plant growth. One possibility is that in *Nmdh*-underexpressing lines the activation states of other redox-modulated chloroplast enzymes are also affected. Fru-1,6-bisphosphatase is highly sensitive to less favorable stromal redox states (Holtgrefe et al., 1997).

Our search for how altered malate-valve capacities might influence the growth of tobacco plants is further complicated by the complex pattern of *Nmdh* expression observed within wild-type plants (Fig. 2). These findings are in accordance with previously published results (Merlo et al., 1993) and indicate that altered *Nmdh* expression in the mutant plants has possibly a more pronounced effect on young, expanding leaves. Here the expression of enzymes such as PEP carboxylase and nitrate reductase is increased, whereas the activities of key enzymes of the Calvin cycle are low (Vivekanandan and Edwards, 1987). In young leaves light energy is predominantly used for anabolic processes such as amino acid synthesis. Moreover, the structural differences between leaves at the various developmental stages might even affect the composition of thylakoid membranes. The chlorophyll-fluorescence characteristics of young leaves often point to effects of photoinhibition, even under low light (Guenther and Melis, 1990). This may indicate that in immature chloroplasts very little capacity is available to adapt light absorption through xanthophyll cycles and chlorophyll-fluorescence-quenching mechanisms. In this situation the increased availability of stromal acceptors might be an advantage, which would be in accordance with the finding that the expression of enzymes involved in the ascorbate-dependent detoxification of O_2 radicals is high in young tobacco leaves (Polle, 1996).

In conclusion, it is obvious that in the transgenic plants described in this study the increase in NADP-MDH capacity correlates with a temporally enhanced growth rate. To understand how this is brought about, more detailed studies are necessary. In particular, the developmental and environmental aspects of NADP-MDH requirements must be considered.

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